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Title: New Simplified Protocols for Timed Artificial Insemination (TAI) in Milk-Producing Donkeys

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Keywords: Keywords: Donkey; artificial insemination; pregnancy rate.

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Abstract: This study compared the outcome of two new timed artificial insemination (TAI) protocols in a milk-producing donkey farm. Ninety Amiata jennies were inseminated at the moment of ovulation induction with hCG, with fresh-transported semen that had been stored at room temperature from 3 up to 6 hours, with an approximate average storage time of 4 hours and a half. In both protocols, on Day 0 jennies were treated with alfaprostol (PGF2α), and on Day 7 they were checked by ultrasound (US) and, if in estrus, they were treated in order to induce ovulation and were then artificially inseminated. In the slow-short TAI protocol, jennies not already inseminated were treated again with PGF2α at Day 14. On day 21 US was repeated and estrus jennies were induced to ovulate and inseminated. In the fast-long TAI protocol, US was performed once a week in jennies not already inseminated and if found in estrus, they were induced to ovulate and inseminated, while those not in estrus were treated again with PGF2α. This protocol was repeated for up to nine weeks. The rates of inseminated/treated, pregnant/inseminated and pregnant/treated jennies were 76%, 56% and 43% for the slow-short TAI protocol and 94%, 47% and 44% for the fast-long TAI protocol. The age class and the lactation status of the jennies had no significant effect on synchronization success or final pregnancy rate. This study demonstrates that it is possible to achieve reasonable pregnancy rates through simplified TAI protocols in jennies, reducing animal handling to a minimum.

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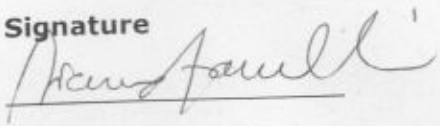
1. that there has been no duplicate publication or submission elsewhere of this work
2. that all authors have read and approved the manuscript, are aware of the submission for publication and agree to be listed as co-authors

Author Name	**Signature**
Diana Fanelli	

***Highlights (for review)**

Highlights

- Ninety milk producing jennies were submitted to Timed Artificial Insemination (TAI), using fresh transported semen, according to a slow-short (21 days) or a fast-long (63 days) protocol.
- The pregnancy rates observed were similar for both TAI protocols, although the long protocol involved a more time-consuming management of jennies.
- The present study demonstrated that it is possible to achieve reasonable pregnancy rates by TAI in jennies using short-time stored semen and reducing animal handling to a minimum.
- This is the first study to report the outcome of AI in donkey using fresh semen stored at room temperature up to 6 hours after collection.

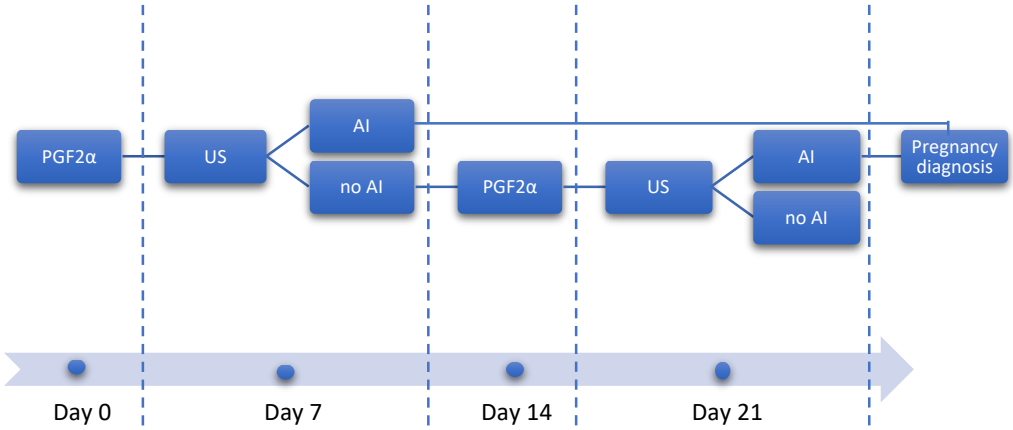
Supplementary files

FIGURE CAPTATION

Figure 1: Slow-short TAI protocol.

Figure 2: Fast-long TAI protocol.

Figure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Pregnancy diagnosis
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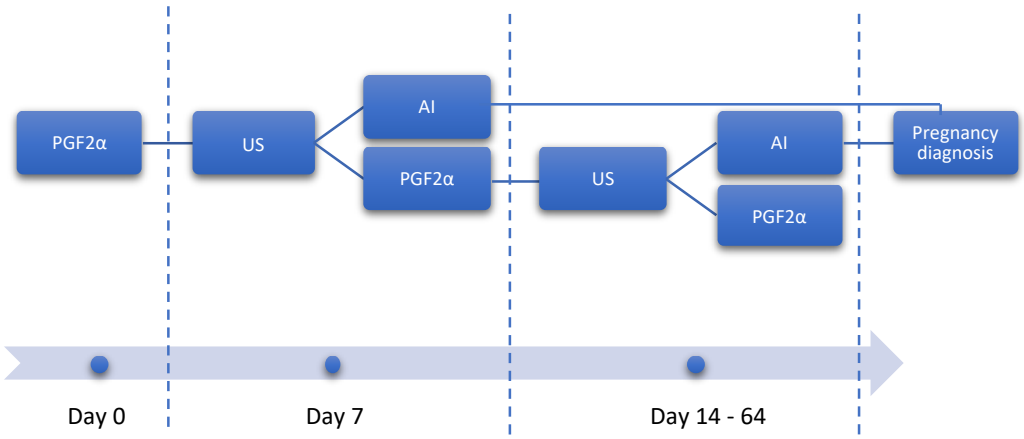
Day 21

Figure


PGF2α
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Pregnancy diagnosis
Day 0
Day 7
Day 14 - 64

Dear reviewers,

thank you for your precious comments.
Please find below answers to your questions.

Yours faithfully
Diana Fanelli

Reviewer 1
THERIO_D_19_00428
Review

Question: The aim of this study was to evaluate two protocols (using alfaprostol and hCG) for timed artificial insemination (TAI) of jennies producing milk for human consumption. The two protocols were tested under field conditions that included insemination, as well as controlled conditions that allowed more intensive monitoring of ovarian activity but did not include insemination. The results of this study add to a fairly small body of literature on the reproductive management of jennies, particularly regarding TAI, and as such warrants publication. One concern I have with the study is the use of hCG as the ovulation inducing agent in the field study versus the use of buserelin in the controlled study, since as the authors note, the response to hCG can be more variable than with a GnRH agonist. In addition, a central premise of this study is to identify hormonal treatment protocols that will be allowable under EU and/or Italian law, which raises the question of whether hCG will remain allowable in the future (particularly since the use of GnRH is not allowed in Italy). Because of that, the potential clinical applicability of the study is questionable.

Answer: At the time of the study the use of GnRH analogues in milk producing jennies was not allowed by the Italian law, but this changed recently and now both hCG and buserelin are both administrable in equids for meat and milk production.

Comments from Reviewer:

- Here are some additional questions/comments I have about the manuscript:

Line Comment

- 34 "above all" can be deleted.
- 67 Replace "implemented" with "supplemented".
- 69 Replace "developed" with "conducted".
- 74 Insert "transrectal" before "ultrasound".
- 127 Use "CL" instead of corpora lutea, since the acronym has already been introduced.
- 148 "AI" instead of "IA".
- 161 I don't see the need to identify individual mares by name (e.g., "AMA").
- 181 "AI" instead of "IA".
- 183 (table) 12/13 = 92% (not 62%).
- 225 "estrous cycle" rather than "estrus cycle".
- 257 As noted previously, please clarify the duration of semen storage.
- 278 Suggest rewording this to "… external and internal components of the reproductive tract …".
- 302 Please ensure that all of the references are formatted correctly.

Answer: All revisions have been made as requested.

Question:

- 11 It is noted that semen was stored for "up to 6 hours". Please provide the minimum storage time and an approximate average storage time.

Answer: Semen had been stored at room temperature from 3 up to 6 hours, with an approximate average storage time of 4 hours and a half. This is now reported in the MS (Lines 16-17).

Question:

- 265: How was "viability" of the spermatozoa determined? If by motility, please state that.

Answer: Authors assume your question is referred to "Oliveira et al. [23] inseminated a few jennies with 1×10^9 or 500×10^6 viable fresh spermatozoa, every 48 hours after the detection of a follicle of 33 to 35 mm in diameter until ovulation detection, with a conception rate of 73% (11/15) and 40% (6/15), respectively." In this paper, (Theriogenology Volume 85, Issue 7, 15 April 2016, Pages 1267-1273) the term "viability" is used but not clearly stated; we just cited what reported by Oliveira. In our paper the quality of ejaculates was evaluated only by motility and sperm number.

Question:

- 204 Regarding the jenny that had "early ovulatory cycles", could this have been due to endometritis causing early luteolysis? Please address this possibility in the discussion.

Answer: In the specific case of the early ovulating jenny from the controlled study, endometritis can be excluded because she was a young fertile animal and her reproductive history was normal. In addition, endometrial cytology was performed with no signs of endometritis.

From the MS, Lines 250-254: the text was revised with "Interestingly, one particular jenny always showed early ovulatory cycles with no endometritis and did not respond to either of the two TAI protocols. In the field studies a similar ovulatory behaviour may explain the lack of response to both TAI protocols although it was not possible to exclude the occurrence of a subclinical endometritis that could lead some jennies the to an early luteolysis."

REVISED HIGHLIGHTED

New Simplified Protocols for Timed Artificial Insemination (TAI) in Milk-Producing Donkeys

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ABSTRACT

This study compared the outcome of two new timed artificial insemination (TAI) protocols in a milk-producing donkey farm. Ninety Amiata jennies were inseminated at the moment of ovulation induction with hCG, with fresh-transported semen that had been stored at room temperature from 3 up to 6 hours, with an approximate average storage time of 4 hours and a half. In both protocols, on Day 0 jennies were treated with alfaprostol (PGF2α), and on Day 7 they were checked by ultrasound (US) and, if in estrus, they were treated in order to induce ovulation and were then artificially inseminated. In the slow-short TAI protocol, jennies not already inseminated were treated again with PGF2α at Day 14. On day 21 US was repeated and estrus jennies were induced to ovulate and inseminated. In the fast-long TAI protocol, US was performed once a week in jennies not already inseminated and if found in estrus, they were induced to ovulate and inseminated, while those not in estrus were treated again with PGF2α. This protocol was repeated for up to nine weeks. The rates of inseminated/treated, pregnant/inseminated and pregnant/treated jennies were 76%, 56% and 43% for the slow-short TAI protocol and 94%, 47% and 44% for the fast-long TAI protocol. The age class and the lactation status of the jennies had no significant effect on synchronization success or final pregnancy rate. This study demonstrates that it is possible to achieve reasonable pregnancy rates through simplified TAI protocols in jennies, reducing animal handling to a minimum.

Keywords: Donkey; artificial insemination; pregnancy rate.

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1. INTRODUCTION

Estrus synchronization and Timed Artificial Insemination (TAI) are tools that improve genetic selection in livestock [1]. Many protocols for estrus synchronization and ovulation have been described in horses [2,3], cows [4–6] and small ruminants [7]. These protocols facilitate the use of TAI in order to manage big livestock herds without estrus detection [4–6].

Although interest in donkey breeding is increasing [8–10], for this species there is still little information in the literature on estrus synchronization and AI. In donkeys, estrus synchronization either by a combination of progesterone and 17-β-estradiol, with two injections of prostaglandin F2α 16 days apart [11], or with other protocols based on the use of prostaglandin F2α analogues and/or altrenogest [12] have been described. In a recent manuscript [13], three TAI protocols based on the use of alfaprostol (a PGF2α-analogue) and GnRH analogues (GnRH) reported pregnancy rates in the inseminated jennies ranging from 11% to 56%.

Due to the risk to human health of hormone residuals in animal products (i.e. meat and milk), the European Union prohibits the use of steroids in farm animals. The use of 17-β-estradiol, testosterone, progesterone, zeranol, trenbolone acetate and melengestrol acetate in farm animals producing milk or meat for human consumption is thus not allowed (Directive 81/602/EEC). In Italy, during this study, the use of GnRH analogues in equids producing food for human consumption was also not allowed.

The aim of this study was to evaluate two protocols for TAI of jennies producing milk for human consumption, both based on the use of alfaprostol and hCG. Protocols were compared in terms of insemination and pregnancy rates, both in the field and in a controlled situation.

2. MATERIALS AND METHODS

The study took place between March and July of two consecutive years (2017-2018) and was approved by the Ethical Committee of the University of Pisa (Prot. n. 45/2017).

2.1 Locations, jennies and management – The two protocols were tested both in field and in controlled conditions. The field study was carried out on a milk donkey farm located in central Italy (42° 53'52.59 N 10°47'05.52 E, WGS84). The farm produces pasteurized milk for human consumption in accordance with the requirements of Regulation (EC) No 853/2004. During the first month of lactation, all the milk is suckled by the foal, while from day 30 after parturition, jennies are machine-milked once daily. Foals are separated from the jennies four hours prior to and during the milking process. A total of 90 non-pregnant Amiata jennies aged 3-20 years, lactating and not lactating, with a body condition score of between 3 and 4 out of 5 [14] were included. Jennies were fed with mixed hay and water ad libitum and, when lactating, the diet was supplemented with about 2.5 kg/day/head of feed for dairy donkeys (Progeo Società Cooperativa Agricola, Reggio Emilia, Italy).

The controlled part of the study was conducted at the Department of Veterinary Science of the University of Pisa, (43° 41′ 00” N, 10° 21′ 00” E) and involved nine cyclic, nonlactating Amiata jennies, aged 6–12 years, with a body condition score of between 3 and 4 out of 5 [14], and an average weight of 280 kg. Jennies were kept in paddocks, fed with hay from mixed-grass meadows and received water *ad libitum*.

The jennies' ovarian activity was monitored by transrectal ultrasound (US) using a machine equipped with a 5-7.5 MHz linear probe (US, Mindray DP30, Shenzhen, China). According to the protocols, jennies were treated with alfaprostol (PGF2α; 1.5 mg, im, Gabbrostim®, Vetem, Spa, Monza-Brianza, Italy) in order to induce luteolysis and a new estrus. Jennies showing a dominant follicle at US of $\geq$ 28 mm of diameter and no evidence of a corpus luteum (CL) were judged to be in estrus, irrespectively of the appearance of the uterine

folds. Jennies with evidence of a CL were judged to be in diestrus, while jennies with neither a CL nor a dominant follicle were judged to be in proestrus. No teaser stallion was used. Ovulation was induced in estrus jennies by hCG (2000 I.U, iv, Vetecor® 5000, Bio98, Milano, Italy) in the field studies, or by GnRH analogue buserelin (GnRH; 1 mg, sc, Suprefact®, Sanofi Spa, Milano) in the controlled studies. In the field, but not in the controlled studies, jennies were submitted to artificial insemination (AI) at the moment of ovulation induction using fresh-transported semen.

2.2 Semen Collection, AI, and Pregnancy Diagnosis – Semen was collected from a fertile jackass stallion, with a body condition score of 3 out of 5 [14], stabled in a box with an outdoor paddock at the Department of Veterinary Sciences, Pisa University, and fed with hay from mixed-grass meadows and water ad libitum.

Semen was collected with a Colorado-model artificial vagina (ARS, Chino, CA), with the aid of an estrus jenny. After collection, semen was filtered through a sterile gauze. Volume and sperm concentration (using a Thoma counting chamber) were then determined, and subjective motility was evaluated after dilution 1:2 in INRA96® (IMV Technologies, France). After evaluation and dilution, semen was transported to the breeding farm in a Styrofoam box at room temperature (20-25°C). AI doses contained $1x10^9$ sperm cells, with an initial subjective motility ranging from 80 up to 95%. Estrus jennies were inseminated only once, in the body of the uterus, at the moment of ovulation induction and within six hours of semen collection. The occurrence of ovulation was not evaluated, and pregnancies were diagnosed by US 21 days after AI.

2.3 Study 1, slow-short TAI (SS-TAI) – In the field study, 54 non-pregnant Amiata jennies aged 3-20 years, lactating (N=33) and not lactating (N=21), were treated with PGF2α on Day 0 and submitted to US on Day 7. Jennies that were evaluated to be in heat were submitted to AI and to ovulation induction with hCG. Jennies not inseminated at Day 7 were treated again with PGF2α on Day 14 and evaluated by US on Day 21. Jennies evaluated to be in heat on Day 21 were submitted to AI and to ovulation induction with hCG on the same day.

Figure 1: Slow-short TAI protocol.

2.4 Study 2, Fast-long TAI (FL-TAI) – In the field study, 36 jennies, lactating (N=18) and not lactating (N=18), were treated with PGF2α on Day 0 and submitted to US on Day 7. If jennies were evaluated to be in heat, AI was performed and ovulation was induced with hCG, as in SS-TAI, while in jennies evaluated as not in heat, PGF2α treatment was repeated immediately. This protocol was repeated weekly for a maximum of nine PGF2α treatments and 10 weeks.

Figure 2: Fast-long TAI protocol.

2.5 Controlled studies – In the two controlled studies, nine jennies were submitted to the same two in field protocols (SS-TAI and FL-TAI) with the following differences: jennies were not inseminated, hCG was replaced by a GnRH analogue to induce ovulation, and US examinations were performed more frequently, starting from Day 0. The aim of this was to follow the ovarian follicle growth, determine the timing of ovulations, and the CL response after PGF2α treatments.

2.6 Statistical Analysis – Fisher's Exact test with Bonferroni correction was used to evaluate the differences in insemination and pregnancy rates in jennies' age classes (≤5, 6-10, 11-15, ≥16 years) and between lactating or not lactating status. GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, La Jolla, CA, www.graphpad.com) was used for statistical analyses. Differences were considered statistically significant when $P<0.05$.

3. RESULTS

3.1 Study 1: SS-TAI – In the field study, the proportion of inseminated and pregnant jennies at the different time points is shown in Table 1, while the proportion in the age classes is reported in Table 2. No statistically significant differences were observed between age classes.

The lactation or non-lactation status had no effects either on the insemination rate (23/33, 70% and 18/21, 86%), nor on the pregnancy rates for the inseminated (12/23, 52% and 11/18, 61%), nor for the treated jennies (12/33, 36% and 11/21, 52%), respectively ($P>0.05$).

N° of PGF2α day	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
1, day 0	17/54 (31)	10/17 (59)	10/54 (19)
2, day 14	24/54 (65)	13/24 (54)	13/54(35)
Total	41/54 (76)	23/41 (56)	23/54 (43)

Table 1: Jennies inseminated and pregnant in slow-short TAI protocol; 13/54 jennies (24%) never met the criteria for insemination. Total length of the protocol = 21 days (day 0 first PGF2α, day 21 last AI)

Age class	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
≤5 years	14/16 (88)	6/14 (43)	6/16 (37)
6-10 years	8/13 (62)	4/8 (50)	4/13 (31)
11-15 years	12/15 (80)	9/12 (75)	9/15 (60)
≥16 years	7/10 (70)	4/7 (57)	4/10 (40)
Total	41/54 (76)	23/41 (56)	23/54 (43)

Table 2: Effect of the age class on insemination and pregnancy rates in slow-short TAI protocol ($P>0.05$).

In the controlled study, the results did not differ from those of the field study, reporting 56% (5/9) and 22% (2/4) of jennies in estrus after the first and the second PGF2α, respectively, and two jennies (22%) that never met the criteria for ovulation induction.

At the timing of the first PGF2α treatment (Day 0), five jennies were in diestrus, three in proestrus and one in estrus.

- 3/5 jennies in diestrus at Day 0 underwent luteolysis, were in estrus at Day 7, and ovulated from 24 to 72 hours after the GnRH treatment;
- 1/5 jennies in diestrus at Day 0 underwent luteolysis, ovulated, and was in diestrus on Day 7 and on Day 14, when she was treated again with PGF2α, underwent luteolysis but was not in estrus on day 21, therefore she never met the criteria for ovulation induction;
- 1/5 jennies in diestrus at Day 0, did not undergo luteolysis, was still in diestrus on Day 7 and on Day 14, when she was treated again with PGF2α and underwent luteolysis, ovulated and was in a new diestrus on Day 21, and therefore never met the criteria for ovulation induction;
- 2/3 jennies in proestrus at Day 0 were in estrus on Day 7 and ovulated 48 hours after the GnRH treatment;
- 1/3 jennies in proestrus and the jenny in estrus at Day 0, were in diestrus on Day 7 and on Day 14, when they were treated again with PGF2α: the two jennies were in estrus on Day 21 and ovulated 48 hours after the GnRH treatment.

3.2 Study 2: FL-TAI – In the field study, the outcomes of the protocol in terms of the inseminated and the pregnant jennies are shown in Table 3, while the results according to age groups are reported in Table 4. After 63 days, 16/18 lactating jennies were inseminated and 5/16 became pregnant, while 18/18 not lactating jennies were inseminated and 11/18 became pregnant. The lactation or not lactation status had no significant effects on insemination rate (89% and 100%, respectively), or on pregnancy rates for inseminated (31% and 61%) or treated jennies (28% and 61%), respectively (P>0.05).

N° of PGF2α, day	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
1, day 0	14/36 (39)	7/14 (50)	7/36 (19)
2, day 7	8/36 (22)	5/8 (63)	5/36 (14)
3, day 14	6/36 (17)	2/6 (33)	2/36 (5)
4, day 21	5/36 (14)	1/5 (20)	1/36 (13)
5 to 8, day 28 to 49	0	-	-
9, day 56	1/36 (3)	1/1 (100)	1/36 (3)
Total	34/36 (94)	16/34 (47)	16/36 (44)

Table 3: Jennies inseminated and pregnant in the fast-long TAI protocol; 2/36 jennies (6%) never met the criteria for insemination. Total length of protocol = 63 days (day 0 first PGF, day 63 last AI)

Age class	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
≤5 years	9/9 (100)	5/9 (56)	5/9 (56)
6-10 years	12/13 (92)	6/12 (50)	6/13 (46)
11-16 years	10/11 (91)	4/10 (40)	4/11 (36)
≥16 years	3/3 (100)	1/3 (33)	1/3 (33)
TOTAL	34/36 (94)	16/34 (47)	16/36 (44)

Table 4: Effect of the age class on insemination and pregnancy rates in fast long TAI protocol (P>0.05).

In the controlled study, the results were very similar to those reported from the field study: after the first, the second and the sixth PGF2α administration, 44% (4/9), 33% (3/9), and 11% (1/9) of the jennies were in heat, respectively. One jenny (11%) never met the criteria for ovulation induction.

In detail, 6 jennies were in diestrus and 3 were in estrus at the time of the first PGF2α (Day 0).

- 4/6 jennies in diestrus at Day 0 underwent luteolysis, were in estrus on Day 7, and ovulated 24-72 hours after the GnRH treatment;
- 2/6 jennies in diestrus at Day 0 did not undergo luteolysis, were still in diestrus on Day 7 when they were treated again with PGF2α. Thus, one jenny underwent luteolysis, was in estrus on Day 14, and ovulated 24 hours after the GnRH treatment, while the other one underwent luteolysis, ovulated and was in diestrus again on Day 14. She was treated with PGF2α, was still in diestrus on Day 21 (CL of day 14), and despite weekly PGF2a treatments, was in diestrus on Day 28 (new CL) and on Day 35 (same CL of day 28). Finally, on Day 42, she was in estrus and ovulated 24 hours after the GnRH treatment.
- 2/3 jennies in estrus at Day 0 were in diestrus on Day 7 when they were treated again with PGF2α. On Day 14 these jennies were in estrus and ovulated 48 hours after the GnRH treatment;
- 1/3 jennies in estrus at Day 0 was in diestrus on Day 7, on Day 14 (new CL), on Day 21 (new CL), on Day 28 (new CL), on Day 35 (previous CL), on Day 42 (new CL), on Day 49 (new CL) and on Day 56 (previous CL). This jenny never met the criteria for GnRH treatment for the induction of ovulation.

4. DISCUSSION

Today, milk from jennies is considered the best choice for the treatment of various diseases in older people [15] and in pediatric medicine for children that are allergic or intolerant to cow milk proteins [8–10]. In cosmetic preparations, jennies' milk is often used as a basic constituent thanks to the fatty-acid antioxidant action and to the high lysozyme content [16]. In addition, The Pan-European Strategy on Biological and Landscape Diversity has promoted pastoral activity and semi-extensive farming due to its positive influence on biodiversity [17]. This thus highlights the importance of preserving autochthonous breeds such as Amiata donkeys and justifies the increased interest in professional donkey breeding.

Jennies are usually pasture or hand bred [18]; however, the use of AI aids genetic selection [1], reduces inbreeding, helps AI in jennies with foal at foot without risks, and can be useful in ensuring milk production throughout the year.

European law (Directive 81/602/EEC) forbids the use of steroid hormones in food-producing animals. In addition, in Italy at the time of this study, the use of GnRH in equids was not allowed. The donkey species has a low economic value, and thus expensive and time-consuming practices are not justified. The present study therefore focused on a protocol for TAI in milk jennies based on the use of PGF2α and hCG and on the minimum impact on animal management.

In donkeys, luteolysis occurs between 15 and 17 days after ovulation [19]. To shorten the estrous cycle, it is possible to use PGF2α from six days after ovulation [20,21] in order to induce a complete luteolysis and a

new estrus cycle. Blanchard [11] reported that after a single or a double, 16 days apart, PGF2α administration, 76% and 73% of jennies, respectively, showed estrus signs with an interval between treatment and estrus of 4.4±1.6 days. In a previous study, we reported estrus synchronization rates from 55 up to 89% after different combinations of PGF2α and GnRH treatments in jennies kept in a controlled environment [13].

In the present study 76% and 94% of the animals were in estrus and underwent AI after the SS-TAI or FL-TAI protocols (lasting 21 and 63 days, respectively). Considering only 21 days for both SS-TAI and FL-TAI protocols, the percentage of inseminated jennies was the same (78 vs 76%, respectively). The FL-TAI protocol resulted in 18% more inseminated jennies but was three times longer than the short one (SS-TAI) and needed more US examinations and PGF2α treatments per jenny (2.6 vs 1.7, respectively). Moreover, in the fast-long TAI protocol, 33/34 of the inseminated jennies were in estrus after the first four PGF2α treatments, while only 1/34 was in estrus only following the 9th PGF2α treatment. Based on these results, continuing the protocol after the fourth PGF2α injection was time consuming and did not considerably increase the number of animals inseminated.

The controlled studies explained why some jennies during the field studies were never in heat despite the PGF2α treatment being repeated once a week for nine weeks in a row. The non-response to TAI protocols was justified by early ovulatory cycles in which, after the PGF2α administration in diestrus, jennies underwent a fast luteolysis, a short estrus and a new ovulation, presenting a new CL seven days after the PGF2α treatment. This new CL, in some cases, was too young to be able to respond properly to the second PGF2α treatment [21], which was still detectable seven days later, when the third PGF2α shot was then performed. In the case of a new early ovulation, the same issue arose again, compromising the possibility of finding these jennies in heat at the fixed time for the ovulation induction. Interestingly, one particular jenny always showed early ovulatory cycles with no endometritis and did not respond to either of the two TAI protocols. In the field studies a similar ovulatory behaviour may explain the lack of response to both TAI protocols although it was not possible to exclude the occurrence of a subclinical endometritis that could lead some jennies the to an early luteolysis.

Studies reporting pregnancy rates after AI in jennies are scarce and mainly focus on the use of frozen [22,23] rather than fresh semen [12,13]. To the best of our knowledge, this is the first study to report the outcome of AI in donkeys using fresh semen stored at 20-25°C for 3 up to 6 hours after collection.

The per cycle pregnancy rate after the use of frozen semen in this species is low (5.5%) [23], and only a few studies report up to 37% if uterine lavage is performed 10 hours post AI [23], or up to 62% with post thawing re-extension of donkey frozen semen with seminal plasma and submitting jennies to an intense ovarian monitoring protocol [22].

The per cycle pregnancy rate after the use of fresh semen in this species is variable. A pregnancy rate of 31% was reported when jennies were treated by GnRH and submitted to AI every 48 hours until the occurrence of ovulation, with one billion fresh spermatozoa [12]. Oliveira et al. [23] inseminated a few jennies with $1x10^9$ or $500x10^6$ viable fresh spermatozoa, every 48 hours after the detection of a follicle of 33 to 35 mm in diameter until ovulation detection, with a conception rate of 73% (11/15) and 40% (6/15), respectively.

A previous study, based on a different combination of PGF2α and GnRH analogue for TAI, reported pregnancy rates of between 17 and 63%, and 11 and 56% per inseminated and per treated jennies, respectively [13].

In the present study, TAI with fresh semen stored for three up to six hours, without ovulation detection, resulted in similar pregnancy rates. In addition, the pregnancy rates observed were similar for the SS-TAI and FL-TAI protocols, both considering the inseminated jennies (56% vs 47%, respectively) and the treated ones (43% and 44%, respectively). Although the long protocol involves a more expensive and time-consuming management of jennies, had no advantages over the short one.

The correlation between advancing age and the decline in fertility is a widespread phenomenon among domestic animals. There is a significant association between mare age and various reproductive measures including reduced oocyte and embryonic viability, degenerative changes in the external and internal components of the reproductive tract and changes in the development of fetal membranes [24]. In the present study, no differences were found when comparing the pregnancy rate of young jennies with older ones. This could be due to the extensive type of reproduction (mostly free-range mating) used for generations, which naturally selected the healthiest and long-living animals. In addition, with our animals this might also have been less stressful for the reproductive tract compared to an intense management typical for mares (e.g. AI with frozen semen or ARTs).

Lactation is the most energy demanding part of parental care in mammals which can reduce the reproductive efficiency in livestock [25], but not in mares [26]. Similarly to equine species, in the present study, lactation was not shown to have a significant influence on insemination or pregnancy rates.

In conclusion, the present study demonstrated that it is possible to achieve reasonable pregnancy rates by subjecting jennies to timed artificial insemination, using short-time stored semen, and reducing animal handling to a minimum. These results could contribute to an improvement in donkey selection for desirable traits through a more extended use of fixed time AI in this species.

Conflicts of interest

The authors declare no conflicts of interest.

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REVISED

New Simplified Protocols for Timed Artificial Insemination (TAI) in Milk-Producing Donkeys

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ABSTRACT

This study compared the outcome of two new timed artificial insemination (TAI) protocols in a milk-producing donkey farm. Ninety Amiata jennies were inseminated at the moment of ovulation induction with hCG, with fresh-transported semen that had been stored at room temperature from 3 up to 6 hours, with an approximate average storage time of 4 hours and a half. In both protocols, on Day 0 jennies were treated with alfaprostol (PGF2α), and on Day 7 they were checked by ultrasound (US) and, if in estrus, they were treated in order to induce ovulation and were then artificially inseminated. In the slow-short TAI protocol, jennies not already inseminated were treated again with PGF2α at Day 14. On day 21 US was repeated and estrus jennies were induced to ovulate and inseminated. In the fast-long TAI protocol, US was performed once a week in jennies not already inseminated and if found in estrus, they were induced to ovulate and inseminated, while those not in estrus were treated again with PGF2α. This protocol was repeated for up to nine weeks. The rates of inseminated/treated, pregnant/inseminated and pregnant/treated jennies were 76%, 56% and 43% for the slow-short TAI protocol and 94%, 47% and 44% for the fast-long TAI protocol. The age class and the lactation status of the jennies had no significant effect on synchronization success or final pregnancy rate. This study demonstrates that it is possible to achieve reasonable pregnancy rates through simplified TAI protocols in jennies, reducing animal handling to a minimum.

Keywords: Donkey; artificial insemination; pregnancy rate.

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1. INTRODUCTION

Estrus synchronization and Timed Artificial Insemination (TAI) are tools that improve genetic selection in livestock [1]. Many protocols for estrus synchronization and ovulation have been described in horses [2,3], cows [4–6] and small ruminants [7]. These protocols facilitate the use of TAI in order to manage big livestock herds without estrus detection [4–6].

Although interest in donkey breeding is increasing [8–10], for this species there is still little information in the literature on estrus synchronization and AI. In donkeys, estrus synchronization either by a combination of progesterone and 17-β-estradiol, with two injections of prostaglandin F2α 16 days apart [11], or with other protocols based on the use of prostaglandin F2α analogues and/or altrenogest [12] have been described. In a recent manuscript [13], three TAI protocols based on the use of alfaprostol (a PGF2α-analogue) and GnRH analogues (GnRH) reported pregnancy rates in the inseminated jennies ranging from 11% to 56%.

Due to the risk to human health of hormone residuals in animal products (i.e. meat and milk), the European Union prohibits the use of steroids in farm animals. The use of 17-β-estradiol, testosterone, progesterone, zeranol, trenbolone acetate and melengestrol acetate in farm animals producing milk or meat for human consumption is thus not allowed (Directive 81/602/EEC). In Italy, during this study, the use of GnRH analogues in equids producing food for human consumption was also not allowed.

The aim of this study was to evaluate two protocols for TAI of jennies producing milk for human consumption, both based on the use of alfaprostol and hCG. Protocols were compared in terms of insemination and pregnancy rates, both in the field and in a controlled situation.

2. MATERIALS AND METHODS

The study took place between March and July of two consecutive years (2017-2018) and was approved by the Ethical Committee of the University of Pisa (Prot. n. 45/2017).

2.1 Locations, jennies and management – The two protocols were tested both in field and in controlled conditions. The field study was carried out on a milk donkey farm located in central Italy (42° 53'52.59 N 10°47'05.52 E, WGS84). The farm produces pasteurized milk for human consumption in accordance with the requirements of Regulation (EC) No 853/2004. During the first month of lactation, all the milk is suckled by the foal, while from day 30 after parturition, jennies are machine-milked once daily. Foals are separated from the jennies four hours prior to and during the milking process. A total of 90 non-pregnant Amiata jennies aged 3-20 years, lactating and not lactating, with a body condition score of between 3 and 4 out of 5 [14] were included. Jennies were fed with mixed hay and water ad libitum and, when lactating, the diet was supplemented with about 2.5 kg/day/head of feed for dairy donkeys (Progeo Società Cooperativa Agricola, Reggio Emilia, Italy).

The controlled part of the study was conducted at the Department of Veterinary Science of the University of Pisa, (43° 41′ 00” N, 10° 21′ 00” E) and involved nine cyclic, nonlactating Amiata jennies, aged 6–12 years, with a body condition score of between 3 and 4 out of 5 [14], and an average weight of 280 kg. Jennies were kept in paddocks, fed with hay from mixed-grass meadows and received water *ad libitum*.

The jennies' ovarian activity was monitored by transrectal ultrasound (US) using a machine equipped with a 5-7.5 MHz linear probe (US, Mindray DP30, Shenzhen, China). According to the protocols, jennies were treated with alfaprostol (PGF2α; 1.5 mg, im, Gabbrostim®, Vetem, Spa, Monza-Brianza, Italy) in order to induce luteolysis and a new estrus. Jennies showing a dominant follicle at US of $\geq$ 28 mm of diameter and no evidence of a corpus luteum (CL) were judged to be in estrus, irrespectively of the appearance of the uterine

folds. Jennies with evidence of a CL were judged to be in diestrus, while jennies with neither a CL nor a dominant follicle were judged to be in proestrus. No teaser stallion was used. Ovulation was induced in estrus jennies by hCG (2000 I.U, iv, Vetecor® 5000, Bio98, Milano, Italy) in the field studies, or by GnRH analogue buserelin (GnRH; 1 mg, sc, Suprefact®, Sanofi Spa, Milano) in the controlled studies. In the field, but not in the controlled studies, jennies were submitted to artificial insemination (AI) at the moment of ovulation induction using fresh-transported semen.

2.2 Semen Collection, AI, and Pregnancy Diagnosis – Semen was collected from a fertile jackass stallion, with a body condition score of 3 out of 5 [14], stabled in a box with an outdoor paddock at the Department of Veterinary Sciences, Pisa University, and fed with hay from mixed-grass meadows and water ad libitum.

Semen was collected with a Colorado-model artificial vagina (ARS, Chino, CA), with the aid of an estrus jenny. After collection, semen was filtered through a sterile gauze. Volume and sperm concentration (using a Thoma counting chamber) were then determined, and subjective motility was evaluated after dilution 1:2 in INRA96® (IMV Technologies, France). After evaluation and dilution, semen was transported to the breeding farm in a Styrofoam box at room temperature (20-25°C). AI doses contained $1x10^9$ sperm cells, with an initial subjective motility ranging from 80 up to 95%. Estrus jennies were inseminated only once, in the body of the uterus, at the moment of ovulation induction and within six hours of semen collection. The occurrence of ovulation was not evaluated, and pregnancies were diagnosed by US 21 days after AI.

2.3 Study 1, slow-short TAI (SS-TAI) – In the field study, 54 non-pregnant Amiata jennies aged 3-20 years, lactating (N=33) and not lactating (N=21), were treated with PGF2α on Day 0 and submitted to US on Day 7. Jennies that were evaluated to be in heat were submitted to AI and to ovulation induction with hCG. Jennies not inseminated at Day 7 were treated again with PGF2α on Day 14 and evaluated by US on Day 21. Jennies evaluated to be in heat on Day 21 were submitted to AI and to ovulation induction with hCG on the same day.

Figure 1: Slow-short TAI protocol.

2.4 Study 2, Fast-long TAI (FL-TAI) – In the field study, 36 jennies, lactating (N=18) and not lactating (N=18), were treated with PGF2α on Day 0 and submitted to US on Day 7. If jennies were evaluated to be in heat, AI was performed and ovulation was induced with hCG, as in SS-TAI, while in jennies evaluated as not in heat, PGF2α treatment was repeated immediately. This protocol was repeated weekly for a maximum of nine PGF2α treatments and 10 weeks.

Figure 2: Fast-long TAI protocol.

2.5 Controlled studies – In the two controlled studies, nine jennies were submitted to the same two in field protocols (SS-TAI and FL-TAI) with the following differences: jennies were not inseminated, hCG was replaced by a GnRH analogue to induce ovulation, and US examinations were performed more frequently, starting from Day 0. The aim of this was to follow the ovarian follicle growth, determine the timing of ovulations, and the CL response after PGF2α treatments.

2.6 Statistical Analysis – Fisher's Exact test with Bonferroni correction was used to evaluate the differences in insemination and pregnancy rates in jennies' age classes (≤5, 6-10, 11-15, ≥16 years) and between lactating or not lactating status. GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, La Jolla, CA, www.graphpad.com) was used for statistical analyses. Differences were considered statistically significant when $P<0.05$.

3. RESULTS

3.1 Study 1: SS-TAI – In the field study, the proportion of inseminated and pregnant jennies at the different time points is shown in Table 1, while the proportion in the age classes is reported in Table 2. No statistically significant differences were observed between age classes.

The lactation or non-lactation status had no effects either on the insemination rate (23/33, 70% and 18/21, 86%), nor on the pregnancy rates for the inseminated (12/23, 52% and 11/18, 61%), nor for the treated jennies (12/33, 36% and 11/21, 52%), respectively ($P>0.05$).

N° of PGF2α day	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
1, day 0	17/54 (31)	10/17 (59)	10/54 (19)
2, day 14	24/54 (65)	13/24 (54)	13/54(35)
Total	41/54 (76)	23/41 (56)	23/54 (43)

Table 1: Jennies inseminated and pregnant in slow-short TAI protocol; 13/54 jennies (24%) never met the criteria for insemination. Total length of the protocol = 21 days (day 0 first PGF2α, day 21 last AI)

Age class	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
≤5 years	14/16 (88)	6/14 (43)	6/16 (37)
6-10 years	8/13 (62)	4/8 (50)	4/13 (31)
11-15 years	12/15 (80)	9/12 (75)	9/15 (60)
≥16 years	7/10 (70)	4/7 (57)	4/10 (40)
Total	41/54 (76)	23/41 (56)	23/54 (43)

Table 2: Effect of the age class on insemination and pregnancy rates in slow-short TAI protocol ($P>0.05$).

In the controlled study, the results did not differ from those of the field study, reporting 56% (5/9) and 22% (2/4) of jennies in estrus after the first and the second PGF2α, respectively, and two jennies (22%) that never met the criteria for ovulation induction.

At the timing of the first PGF2α treatment (Day 0), five jennies were in diestrus, three in proestrus and one in estrus.

- 3/5 jennies in diestrus at Day 0 underwent luteolysis, were in estrus at Day 7, and ovulated from 24 to 72 hours after the GnRH treatment;
- 1/5 jennies in diestrus at Day 0 underwent luteolysis, ovulated, and was in diestrus on Day 7 and on Day 14, when she was treated again with PGF2α, underwent luteolysis but was not in estrus on day 21, therefore she never met the criteria for ovulation induction;
- 1/5 jennies in diestrus at Day 0, did not undergo luteolysis, was still in diestrus on Day 7 and on Day 14, when she was treated again with PGF2α and underwent luteolysis, ovulated and was in a new diestrus on Day 21, and therefore never met the criteria for ovulation induction;
- 2/3 jennies in proestrus at Day 0 were in estrus on Day 7 and ovulated 48 hours after the GnRH treatment;
- 1/3 jennies in proestrus and the jenny in estrus at Day 0, were in diestrus on Day 7 and on Day 14, when they were treated again with PGF2α: the two jennies were in estrus on Day 21 and ovulated 48 hours after the GnRH treatment.

3.2 Study 2: FL-TAI – In the field study, the outcomes of the protocol in terms of the inseminated and the pregnant jennies are shown in Table 3, while the results according to age groups are reported in Table 4. After 63 days, 16/18 lactating jennies were inseminated and 5/16 became pregnant, while 18/18 not lactating jennies were inseminated and 11/18 became pregnant. The lactation or not lactation status had no significant effects on insemination rate (89% and 100%, respectively), or on pregnancy rates for inseminated (31% and 61%) or treated jennies (28% and 61%), respectively (P>0.05).

N° of PGF2α, day	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
1, day 0	14/36 (39)	7/14 (50)	7/36 (19)
2, day 7	8/36 (22)	5/8 (63)	5/36 (14)
3, day 14	6/36 (17)	2/6 (33)	2/36 (5)
4, day 21	5/36 (14)	1/5 (20)	1/36 (13)
5 to 8, day 28 to 49	0	-	-
9, day 56	1/36 (3)	1/1 (100)	1/36 (3)
Total	34/36 (94)	16/34 (47)	16/36 (44)

Table 3: Jennies inseminated and pregnant in the fast-long TAI protocol; 2/36 jennies (6%) never met the criteria for insemination. Total length of protocol = 63 days (day 0 first PGF, day 63 last AI)

Age class	Inseminated/total (%)	Pregnant/inseminated (%)	Pregnant/total (%)
≤5 years	9/9 (100)	5/9 (56)	5/9 (56)
6-10 years	12/13 (92)	6/12 (50)	6/13 (46)
11-16 years	10/11 (91)	4/10 (40)	4/11 (36)
≥16 years	3/3 (100)	1/3 (33)	1/3 (33)
TOTAL	34/36 (94)	16/34 (47)	16/36 (44)

Table 4: Effect of the age class on insemination and pregnancy rates in fast long TAI protocol (P>0.05).

In the controlled study, the results were very similar to those reported from the field study: after the first, the second and the sixth PGF2α administration, 44% (4/9), 33% (3/9), and 11% (1/9) of the jennies were in heat, respectively. One jenny (11%) never met the criteria for ovulation induction.

In detail, 6 jennies were in diestrus and 3 were in estrus at the time of the first PGF2α (Day 0).

- 4/6 jennies in diestrus at Day 0 underwent luteolysis, were in estrus on Day 7, and ovulated 24-72 hours after the GnRH treatment;
- 2/6 jennies in diestrus at Day 0 did not undergo luteolysis, were still in diestrus on Day 7 when they were treated again with PGF2α. Thus, one jenny underwent luteolysis, was in estrus on Day 14, and ovulated 24 hours after the GnRH treatment, while the other one underwent luteolysis, ovulated and was in diestrus again on Day 14. She was treated with PGF2α, was still in diestrus on Day 21 (CL of day 14), and despite weekly PGF2a treatments, was in diestrus on Day 28 (new CL) and on Day 35 (same CL of day 28). Finally, on Day 42, she was in estrus and ovulated 24 hours after the GnRH treatment.
- 2/3 jennies in estrus at Day 0 were in diestrus on Day 7 when they were treated again with PGF2α. On Day 14 these jennies were in estrus and ovulated 48 hours after the GnRH treatment;
- 1/3 jennies in estrus at Day 0 was in diestrus on Day 7, on Day 14 (new CL), on Day 21 (new CL), on Day 28 (new CL), on Day 35 (previous CL), on Day 42 (new CL), on Day 49 (new CL) and on Day 56 (previous CL). This jenny never met the criteria for GnRH treatment for the induction of ovulation.

4. DISCUSSION

Today, milk from jennies is considered the best choice for the treatment of various diseases in older people [15] and in pediatric medicine for children that are allergic or intolerant to cow milk proteins [8–10]. In cosmetic preparations, jennies' milk is often used as a basic constituent thanks to the fatty-acid antioxidant action and to the high lysozyme content [16]. In addition, The Pan-European Strategy on Biological and Landscape Diversity has promoted pastoral activity and semi-extensive farming due to its positive influence on biodiversity [17]. This thus highlights the importance of preserving autochthonous breeds such as Amiata donkeys and justifies the increased interest in professional donkey breeding.

Jennies are usually pasture or hand bred [18]; however, the use of AI aids genetic selection [1], reduces inbreeding, helps AI in jennies with foal at foot without risks, and can be useful in ensuring milk production throughout the year.

European law (Directive 81/602/EEC) forbids the use of steroid hormones in food-producing animals. In addition, in Italy at the time of this study, the use of GnRH in equids was not allowed. The donkey species has a low economic value, and thus expensive and time-consuming practices are not justified. The present study therefore focused on a protocol for TAI in milk jennies based on the use of PGF2α and hCG and on the minimum impact on animal management.

In donkeys, luteolysis occurs between 15 and 17 days after ovulation [19]. To shorten the estrous cycle, it is possible to use PGF2α from six days after ovulation [20,21] in order to induce a complete luteolysis and a

new estrus cycle. Blanchard [11] reported that after a single or a double, 16 days apart, PGF2α administration, 76% and 73% of jennies, respectively, showed estrus signs with an interval between treatment and estrus of 4.4±1.6 days. In a previous study, we reported estrus synchronization rates from 55 up to 89% after different combinations of PGF2α and GnRH treatments in jennies kept in a controlled environment [13].

In the present study 76% and 94% of the animals were in estrus and underwent AI after the SS-TAI or FL-TAI protocols (lasting 21 and 63 days, respectively). Considering only 21 days for both SS-TAI and FL-TAI protocols, the percentage of inseminated jennies was the same (78 vs 76%, respectively). The FL-TAI protocol resulted in 18% more inseminated jennies but was three times longer than the short one (SS-TAI) and needed more US examinations and PGF2α treatments per jenny (2.6 vs 1.7, respectively). Moreover, in the fast-long TAI protocol, 33/34 of the inseminated jennies were in estrus after the first four PGF2α treatments, while only 1/34 was in estrus only following the 9^{th} PGF2α treatment. Based on these results, continuing the protocol after the fourth PGF2α injection was time consuming and did not considerably increase the number of animals inseminated.

The controlled studies explained why some jennies during the field studies were never in heat despite the PGF2α treatment being repeated once a week for nine weeks in a row. The non-response to TAI protocols was justified by early ovulatory cycles in which, after the PGF2α administration in diestrus, jennies underwent a fast luteolysis, a short estrus and a new ovulation, presenting a new CL seven days after the PGF2α treatment. This new CL, in some cases, was too young to be able to respond properly to the second PGF2α treatment [21], which was still detectable seven days later, when the third PGF2α shot was then performed. In the case of a new early ovulation, the same issue arose again, compromising the possibility of finding these jennies in heat at the fixed time for the ovulation induction. Interestingly, one particular jenny always showed early ovulatory cycles with no endometritis and did not respond to either of the two TAI protocols. In the field studies a similar ovulatory behaviour may explain the lack of response to both TAI protocols although it was not possible to exclude the occurrence of a subclinical endometritis that could lead some jennies the to an early luteolysis.

Studies reporting pregnancy rates after AI in jennies are scarce and mainly focus on the use of frozen [22,23] rather than fresh semen [12,13]. To the best of our knowledge, this is the first study to report the outcome of AI in donkeys using fresh semen stored at 20-25°C for 3 up to 6 hours after collection.

The per cycle pregnancy rate after the use of frozen semen in this species is low (5.5%) [23], and only a few studies report up to 37% if uterine lavage is performed 10 hours post AI [23], or up to 62% with post thawing re-extension of donkey frozen semen with seminal plasma and submitting jennies to an intense ovarian monitoring protocol [22].

The per cycle pregnancy rate after the use of fresh semen in this species is variable. A pregnancy rate of 31% was reported when jennies were treated by GnRH and submitted to AI every 48 hours until the occurrence of ovulation, with one billion fresh spermatozoa [12]. Oliveira et al. [23] inseminated a few jennies with $1x10^9$ or $500x10^6$ viable fresh spermatozoa, every 48 hours after the detection of a follicle of 33 to 35 mm in diameter until ovulation detection, with a conception rate of 73% (11/15) and 40% (6/15), respectively.

A previous study, based on a different combination of PGF2α and GnRH analogue for TAI, reported pregnancy rates of between 17 and 63%, and 11 and 56% per inseminated and per treated jennies, respectively [13].

In the present study, TAI with fresh semen stored for three up to six hours, without ovulation detection, resulted in similar pregnancy rates. In addition, the pregnancy rates observed were similar for the SS-TAI and FL-TAI protocols, both considering the inseminated jennies (56% vs 47%, respectively) and the treated ones (43% and 44%, respectively). Although the long protocol involves a more expensive and time-consuming management of jennies, had no advantages over the short one.

The correlation between advancing age and the decline in fertility is a widespread phenomenon among domestic animals. There is a significant association between mare age and various reproductive measures including reduced oocyte and embryonic viability, degenerative changes in the external and internal components of the reproductive tract and changes in the development of fetal membranes [24]. In the present study, no differences were found when comparing the pregnancy rate of young jennies with older ones. This could be due to the extensive type of reproduction (mostly free-range mating) used for generations, which naturally selected the healthiest and long-living animals. In addition, with our animals this might also have been less stressful for the reproductive tract compared to an intense management typical for mares (e.g. AI with frozen semen or ARTs).

Lactation is the most energy demanding part of parental care in mammals which can reduce the reproductive efficiency in livestock [25], but not in mares [26]. Similarly to equine species, in the present study, lactation was not shown to have a significant influence on insemination or pregnancy rates.

In conclusion, the present study demonstrated that it is possible to achieve reasonable pregnancy rates by subjecting jennies to timed artificial insemination, using short-time stored semen, and reducing animal handling to a minimum. These results could contribute to an improvement in donkey selection for desirable traits through a more extended use of fixed time AI in this species.

Conflicts of interest

The authors declare no conflicts of interest.

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